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13. ABSTRACT (Maximum 200 Words)

The core of my hypothesis is that psoriasin, a protein shown to be aberrantly expressed at different stages of breast cancer, physically interacts with hGCP3, an indispensable component of the •-tubulin ring complex involved in centrosome function.
Aims for the first year of the project were to determine if a Histidine-tagged hGCP3 (His-hGCP3) construct previously generated in our lab is biologically suitable for this study and to substantiate an interaction between psoriasin and hGCP3 in mammalian cells using immunofluorescent co-localization and biochemical co-immunoprecipitation (co-IP) assays.
In the first year I have shown that the His-tag does not interfere with His-hGCP3 ability to interact with •-tubulin by co-IP assays; that expression of this construct does have a mild cytotoxic effect but is not completely lethal; that available psoriasin antibodies are not suitable for IF labeling therefore preventing co-localization studies with hGCP3, that psoriasin and His-hGCP3 cannot be consistently co-IP'ed suggesting at best, a weak or a possible cell-cycle dependent interaction exists. Using two assays to measure centrosome function, a quantitative assay for microtubule (MT) nucleation and a mitotic spindle morphology assay, I've further ascertained that psoriasin expression in does not alter centrosome function.

14. SUBJECT TERMS
Molecular pathology, cell biology, s100A7, hGCP3, centrosome, microtubule, transient transfection, immunofluorescence, immunoprecipitation

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Introduction

The core of my hypothesis is that psoriasin, a member of the S100A family of proteins shown to be aberrantly expressed at different stages of breast cancer, physically interacts with hGCP3. hGCP3 is an indispensable component of a much larger protein complex, the γ-tubulin ring complex (γTuRC), and is intrinsically involved in centrosome function. Therefore we further hypothesise this interaction has downstream effects on centrosome function; moreover, altered psoriasin expression is able to influence cellular properties associated with cancer progression, in part, through a physical interaction with hGCP3.

The aim of the first year of the project was first to determine if a Histidine-tagged hGCP3 (His-hGCP3) construct previously generated in our lab is biologically suitable for this study; the epitope-tagged hGCP3 must behave like endogenous hGCP3, and expression of this construct must not be lethal to the cell. Additional aims for the first year were to substantiate an interaction between psoriasin and hGCP3 in mammalian cells using immunofluorescent co-localization and biochemical co-immunoprecipitation (co-IP) assays. Furthermore, additional aims that span the first and second year are to generate an inducible MCF-7 breast cancer cell model that expresses the His-tagged hGCP3 using the Tet-On system from Clontech.

In the first year I have shown that the His-tag does not interfere with His-hGCP3 ability to interact with γ-tubulin by co-IP assays; that expression of this construct dose have a mild cytotoxic effect but is not completely lethal; that available psoriasin antibodies are not suitable for IF labelling therefore preventing co-localization studies with hGCP3, that psoriasin and His-hGCP3 cannot be consistently co-IP'ed. This may suggest that the interaction identified is a false positive, or is peculiar to the conditions of the yeast 2-hybrid assay, or may possibly be a cell-cycle dependent interaction. Moreover I have begun to generate an inducible His-hGCP3 MCF-7 model. In the interim, I've directly pursued specific aims of Task 2B proposed in statement-of-work (SOW) for months 15-23, this year. Using two assays to measure centrosome function, a quantitative assay for microtubule (MT) nucleation and a mitotic spindle morphology assay, I've ascertained that psoriasin expression does not alter centrosome function as determined by these assays.

Body

Task 1: To Determine if hGCP3 and Psoriasin Physically Interact

i. To Determine if epitope-tagged hGCP3 behaves like endogenously expressed hGCP3

We have generated an amino-terminal histidine-tagged hGCP3 construct (His-hGCP3) to test whether hGCP3 and psoriasin physically interact in mammalian cells. However, it is paramount that His-tag does not interfere with normal hGCP3 behaviour in order for subsequent results to merit credit. To test this, co-IP assays were performed to determine if His-hGCP3 to retains the

ability to interact with γ-tubulin, as is reported in the literature for its endogenous counterpart (Murphy SM et al., 1998; Tassin AM et al., 1998).

Briefly, equal volume of whole-cell lysates from Cos1 cells that were transiently transfected with either His-HGCP3 or empty vector (pcDNA4His/Max, Invitrogen), were immunoprecipitated with either Anti-His (Invitrogen), Anti-γ-tubulin (Sigma), or control IgG antibodies. Western analysis with anti-His antibody detected His-HGCP3 in γ-tubulin immunoprecipitates from cells transfected with His-hGCP3 (Figure 1A, Lanes 3-4) but not empty vector (Figure1A, Lane 1). Control antibody immunoprecipitates (Figure 1A, Lane 2) did not contain His-HGCP3 inferring specificity of γ-tubulin antibody. A non specific band migrating faster than His-hGCP3 was always detected with the anti-His antibody irrespective of transfection (Figure1A, Lane 5) and serves as an internal loading control for subsequent experiments. All immunoprecipitates were clean as indicated by the absence of the non-specific band present in input lanes (Figure 1A, lanes 5-8). Conversely, western analysis with anti-γ-Tubulin antibody detected γ-tubulin in anti-His-immunoprecipitates (Figure 1B, Lane 2) from cells transfected with His-hGCP3. Although a band corresponding to γ-tubulin is detected in the control-antibody-IP lane (Figure 1B, Lane 1), it is significantly weaker than Anti-His-IP and input lanes (Figure 1B, Lane 3-4).

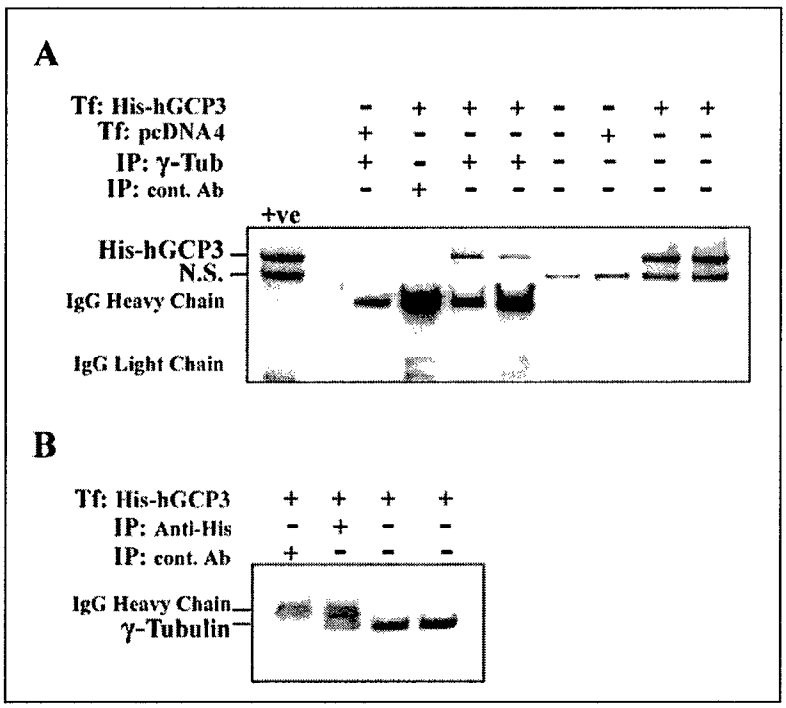

Figure 1: N-terminal Histidine tag does not interfere with His-hGCP3's γ-Tubulin interacting properties in transiently transfected Cos1 cells. (A) γ-tubulin immunoprecipitates contain His-hGCP3, while control IP'ed, and mock transfected (pcDNA4His/Max) do not (see text for details). (B) Conversely, γ-tubulin is detected in anti-His immunoprecipitates in His-hGCP3 transfected cells. (Tf, transfected; pcDNA4, pcDNA4His/Max; γ-tub, γ-tubulin; cont. Ab, control antibody; N.S., non specific)

These results suggest that the N-terminal His-tag does not prevent His-hGCP3 from associating with endogenous γ-tubulin, and indicates that the His-HGCP3 behaves like it endogenous counterpart.

ii. Characterize viability of transient transfectants to ensure interaction are occurring in viable cells

Overexpression of other GCPs has been shown to be toxic in a variety of cells (Fava F et al., 1999; Geissler S et al., 1996; Murphy SM et al., 1998). This immediately raised the concern that overexpression of His-hGCP3 may prove to have the same effect considering hGCP3s indispensable role within the γ-TuRC. To assess the effect of His-hGCP3 overexpression in mammalian cells, Cos1 cells were transiently transfected with either His-hGCP3 or empty vector (pcDNA4His/Max, Invitrogen). Levels of His-hGCP3 expression were monitored by western blot analysis, and cellular toxicity was inferred by morphological assessment of cells with phase contrast microscopy.

Phase contrast microscopy 24hr post-transfection revealed that overall Cos1 cell populations transfected with His-hGCP3 were less confluent than control plates, and cells adjacent to 'clearings' presented an emaciated, spindly morphology. In contrast, control cells transfected with the empty vector appeared significantly healthier and were confluent 24h after transfection with cuboidal cell morphology, likely reflecting compressed confluent cell environment (Figure 2A). Western analysis of the adherent portion of cells with anti-His antibody revealed a robust His-GCP3 signal 24h post transfection in His-hGCP3 transfected cells but not in control transfected samples (Figure2B). A reduction in His-GCP3 expression is observed 72h post-transfection with no change in the non-specific band intensity.

Taken together these results suggest that robust overexpression of His-hGCP3 is likely to have immediate toxic effects. However, persistent expression of His-hGCP3 72h after transfection suggests that a large portion of transfected cells remain in the adherent population indicating that His-hGP3 expression (likely at modest levels) is not lethal. This reduction in the expression may reflect cellular toxicity or growth inhibition, thereby allowing non-transfected cells to out-grow and effectively dilute His-hGCP3 protein concentration in whole cell lysates. Alternatively, this reduction may simply reflect increased protein turnover combined with decreased production likely through plasmid degradation, a common phenomena in transiently transfected cells.

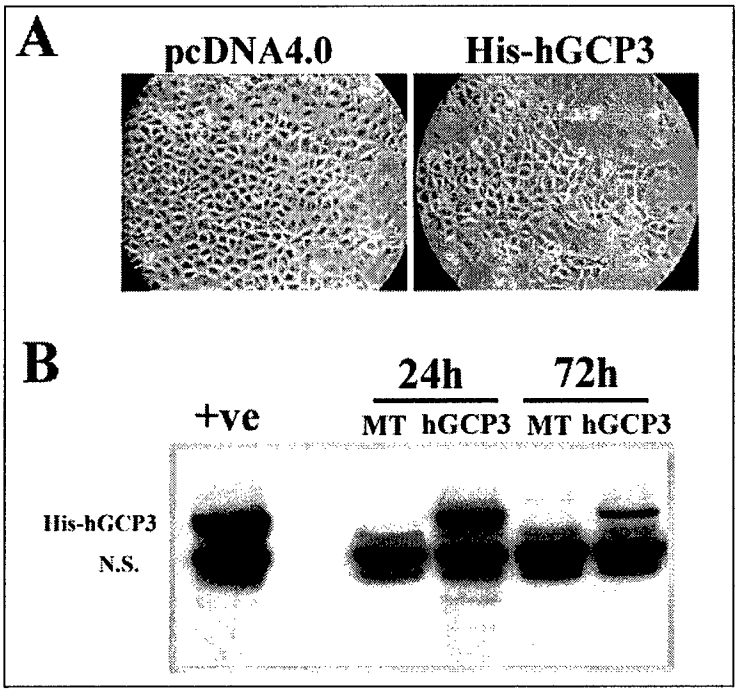

Figure 2: Robust His-hGCP3 expression has immediate cytotoxic effects, but significant portions of cells with modest expression persist in the population 72 hours post-transfection. (A) Representative phase contrast images (400X) of Cos1 cells 24 hours post transfection, showing clear signs of toxicity associated with His-hGCP3 expression. (B) Representative western blot showing reduction, but persistent His-hGCP3 expression 72 hours post transfection. (MT, mock transfection; h, hours; N.S., non specific)

iii. Co-localization of psoriasin with hGCP3.

First it was necessary to determine if available anti-psoriasin antibodies would be suitable for indirect immunofluorescence (IF). Cold methanol fixation is a preferred method of fixation when microtubule and centrosome structures are visualized by IF microscopy as it is rapid and provides excellent structure preservation. This method is typically used when visualizing centrosomal localized proteins including γ-tubulin and pericentrin. Therefore, to determine if psoriasin and hGCP3 co-localize, IF staining and microscopy on cold methanol fixed cells was used. To test antibody specificity, two psoriasin-overexpressing cell models were used from Hela and MB-MDA-231 cell origin that were previously engineered in our lab. Two anti-Psoriasin antibodies were tested for their ability to specifically label psoriasin for indirect-IF under a variety of conditions. The first antibody, a rabbit-polyclonal (RP) antibody that recognises 14-amino acid peptide corresponding to carboxy-terminus of psoriasin (KQSHGAAPCSGGSQ) was previously developed and characterized in our lab (Emberley ED et al., 2003) (Figure 3A). The second antibody tested is commercially available mouse monoclonal (MM) IgG raised against full-length psoriasin (AbCam) (Figure 3B). Psoriasin expression was monitored by western analysis showing no psoriasin expression in Hela.12 or 231-1A1s, but robust expression in

Hela.16 and 231-Fa3. In addition, many non-specific bands are detected by western analysis with RP anti-psoriasin antibody, while a single band, corresponding to psoriasin, is detected with the MM antibody.

Unfortunately, neither anti-psoriasin antibody tested showed psoriasin-specificity as detected by indirect-IF labelling. Both the intensity and cellular distribution of signal remained the same irrespective of the presence of psoriasin and under any variety of experimental conditions used. Tested conditions included: different 1^0 and 2^0 antibody concentration, with or without pre-extraction of soluble proteins before fixation, or with acetone or mild detergent (0.01% TritonX-100) permeabilisation post fixation (data not shown). The non-specific labelling is attributable to 1^0 antibody, as omission controls reveal no signal for the RP antibody (Figure3A, top panel) or reduced signal for the MM antibody (Figure3B, top panel). RP had an overall higher background fluorescent intensity, likely a reflection of the many non-specific bands detected by western analysis. These results indicate that neither antibody is suitable for indirect-IF and as such pose a significant obstacle for co-localization studies.

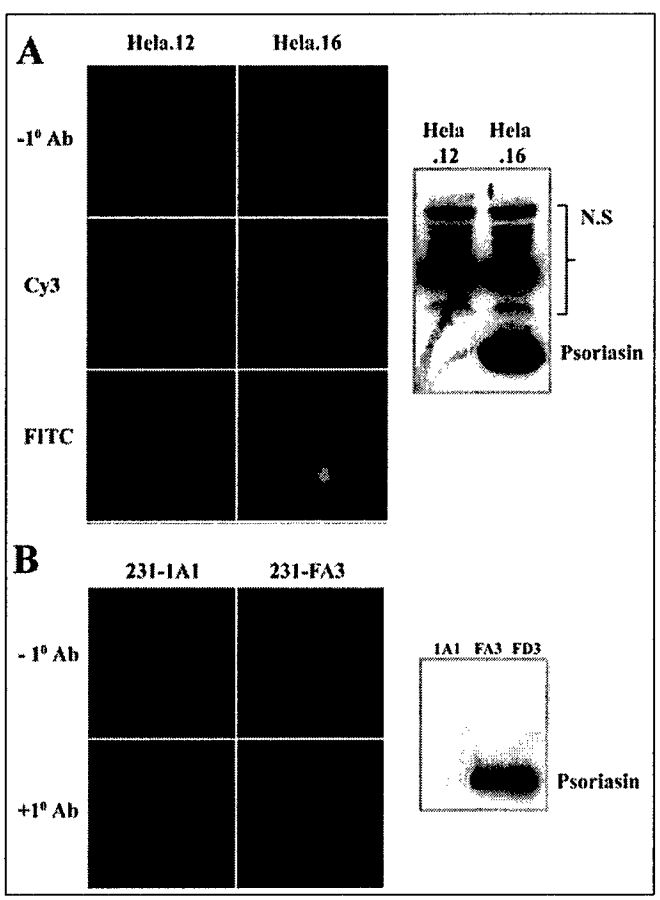

Figure 3: Anti-Psoriasin antibodies tested in this study are not suitable for indirect-IF. Antibodies tested include (A) in-lab rabbit-polyclonal antibody and (B) commercially available mouse monoclonal antibody (Abcam). For a given anti-psoriasin antibody the intensity and cellular distribution of signal remained the same irrespective of the presence of psoriasin (left versus right columns) as monitored by western analysis (Right panels).

iv. Determine if Psoriasin physically interact by co-immunoprecipitation in transiently transfected cells

Previous results from a yeast-two-hybrid screen performed in our lab indicated an interaction between hGCP3 and psoriasin can occur. To substantiate this observation but in a mammalian cell, co-immunoprecipitation analysis was performed on transiently co-transfected Cos1 cells. Cos1 cell were transfected with His-hGCP3 as well as a construct expressing psoriasin (pcDNA3.1-psor). Equal volumes of co-transfected Cos1 cell lysates were immunoprecipitated with either Anti-His or control mouse monoclonal IgG antibodies followed by western analysis with an anti-psoriasin antibody. Despite using several different transfection and IP conditions, it was not possible to consistently detect psoriasin in anti-His immunoprecipitates. In one experiment, faint bands corresponding to psoriasin were detected in the immunoprecipitates of Cos1 cells co-transfected with either equal molar (Figure 4A, Lane3) or equal ug (Figure 4A, Lane 5) amounts of plasmid DNA, but not in the immunoprecipitate of an unrelated mmIgG antibody (Figure 4A, Lane 2). More often than not however, anti-His immunoprecipitates did not contain psoriasin (Figure 4B, Lane 5-6), even upon robust co-expression of psoriasin and His-hGCP3 (Figure 4B, Lane 1-3).

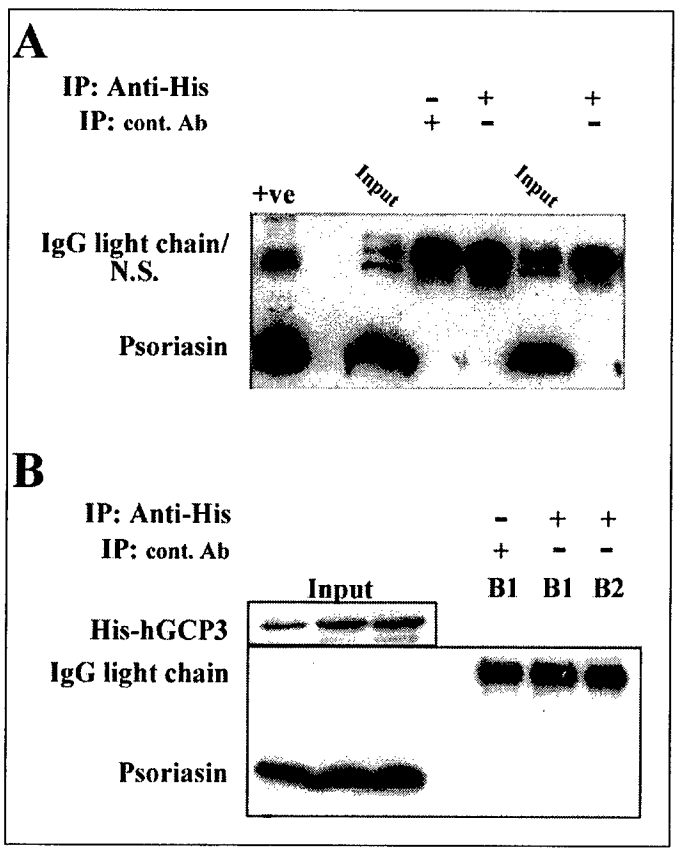

Figure 4: Representative Co-IPs showing (A) positive Psoriasin/ His-hGCP3 interaction and (B) negative Psoriasin/ His-hGCP3 interaction. (A) In one experiment, psoriasin was detected in His-hGCP3 immunoprecipitates of Cos1 cells co-transfected with either equal molar (Lane3) or equal ug (Lane 5) of His-hGCP3 and pcDNA3.1-psor plasmids. (B) In other cases, despite robust His-hGCP3 and psoriasin expression, His-hGCP3 immunoprecipitates did not contain psoriasin (Lane 5-6). (IP, immunoprecipitating antibody; B1, co-IP buffer one; B2, co-IP buffer two)

These results suggest one of two possibilities. First, positive co-IP are due to experimental error and psoriasin and hGCP3 do not interact. It certainly appears that sample cross-contamination or lane-bleeding may very well explain results in Figure 4A. Alternatively, an interaction does occur, however, the interaction is not kinetically favoured under experimental conditions, or takes places at specific stage in the cell-cycle that was not controlled for in subsequent negative co-IPs. However, having failed to reproduce a positive interaction by co-immunoprecipitation the requisite experimental three-times, it must be concluded that psoriasin and His-hGCP3 do not interact under experimental conditions.

Task 2: Assay For Biological Effects Modulated by a hGCP3/Psoriasin Interaction

As a result of the inability to consistently co-IP His-hGCP3 and psoriasin, I've directly pursued specific aims of Task 2B proposed in statement-of-work (SOW) for months 15-23, this year. The question of whether psoriasin overexpression has an effect on centrosomal function mediated through hGCP3 is at the heart of this proposal's relevance to breast cancer research. In this way, an overall conclusion to whether a psoriasin-hGCP3 interaction is biologically significant in breast cancer may be ascertained more rapidly than the accepted SOW timeline.

To date, no specific assay has been developed to measure hGCP3 function directly. In order to determine if psoriasin (over)expression has a biological effect on hGCP3 function, it was rationalized that as an indispensable and intrinsic component of γTuRC, modulation of hGCP3 function would likely affect γTuRC function therefore translate into altered centrosome function. Therefore centrosome function was determined using a quantitative assay for microtubule (MT) nucleation and mitotic spindle morphology.

i. **To determine if Psoriasin expression has an effect on Mitotic Spindle Morphology (MSM)**

To determine if aberrant psoriasin expression has a biological effect on mitotic spindle formation, spindle morphology was assessed by IF microscopy in two psoriasin-expressing epithelial breast cancer cell models previously engineered in the lab. Mitotic spindles were visualised in methanol fixed (-20^{0}C) / acetone permeabilised cells using direct IF labelling of β-tubulin with a Cy3-anti-β-tubulin antibody (Sigma). Dapi staining was used to visualise DNA. Spindle defects that were scored included monopolar, multipolar, misaligned and unknown defects (Figure 5A). For this study a psoriasin-inducible MCF7 model (Figure 5B) and a constitutive psoriasin expression MDA-MB-231 model (Figure5C) were used. Both the frequency and type of aberrant spindles were scored in three separate experiments, in which 100 consecutively viewed metaphase or anaphase spindles were scored per cell line used.

Levels of psoriasin expression were monitored by western analysis with anti-psoriasin antibodies (RP or the MM (Abcam)). Shown are representative western blots revealing no psoriasin expression in wild type MCF7, but significant induction of psoriasin expression in the MCF7-MAG clones (Mag06 and Mag07) after 48hr of doxycycline treatment (Figure 5B, Lower Panel). Moreover, no psoriasin expression is detected in wild type MDA-MB-231, or in the 1a1 clone, but robust psoriasin-expression is detected in the Fa3 and Fd3 clones (Figure 5C, Lower Panel).

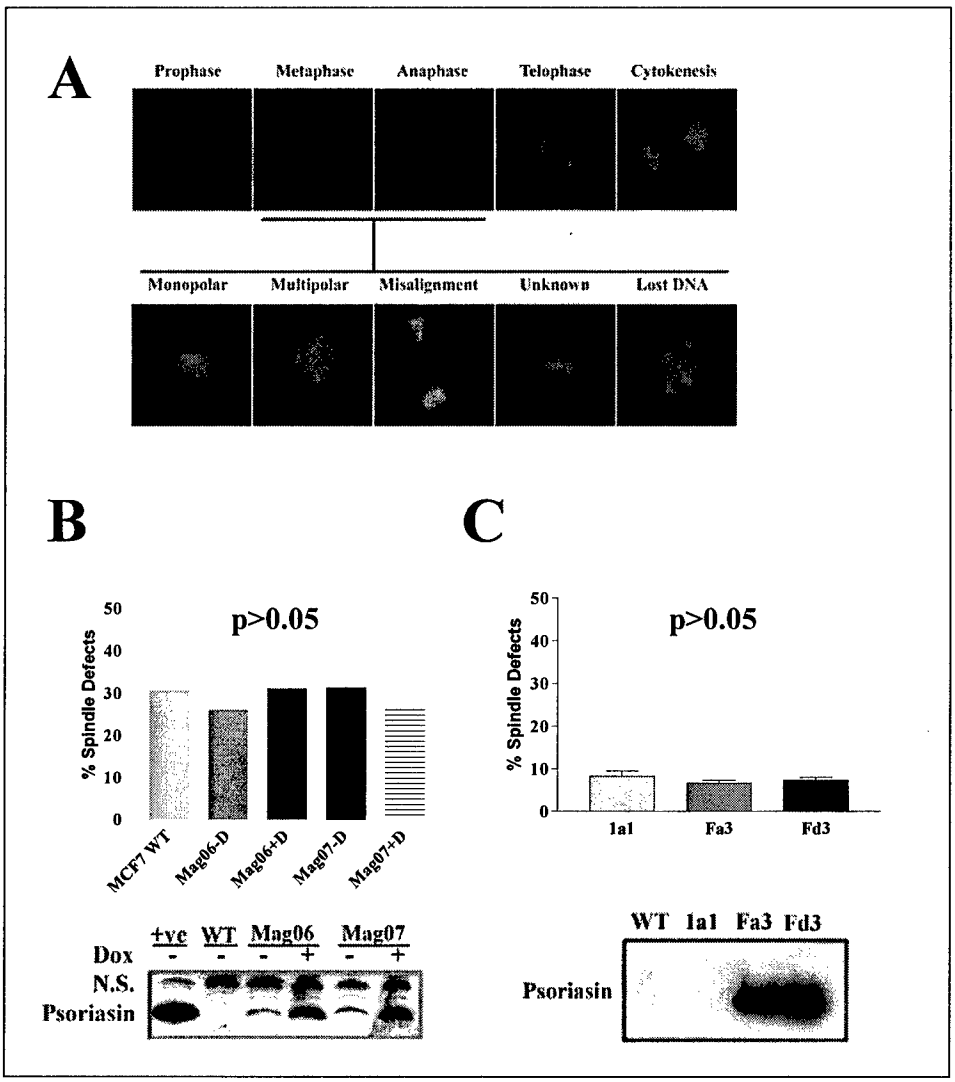

Figure5: Psoriasin expression does not change the frequency of mitotic spindle defects. (A) Mitotic spindle morphology changes throughout mitosis (Top panel) and a range of specific types of spindle defects are observed in metaphase and anaphase cells (Bottom Panel). (B) Frequency of spindle defects (~30%) did not change upon psoriasin induction with doxycycline in MCF7-Mag clones as compared to the non-induced ($p>0.05$, Anova) and wild-type MCF7 ($p>0.05$, Anova). (C) Same results were obtained with MDA-MB-231 model, however the total frequency of spindle defects observed were significantly lower (~10%). Bars represent the mean +/- S.D. Western blot analysis was used to monitor psoriasin expression (B and C, Lower panel). (D or Dox, doxycycline)

Analysis of mitotic defects revealed that wild-type MCF7 and derived clones had an overall higher frequency of spindle defects (~30%) (Figure 5B, upper panel) than in the 231s and derived clones (~10%) (Figure 5C, upper panel). In both cell lines however, psoriasin overexpression did not affect the frequency of mitotic spindle defects or the frequency of specific types of defects analysed ($p>0.05$, Anova test). Interestingly, roughly 80% of total spindle defects observed in MCF7 were monopolar spindles, while 231s presented a combination of multi-polar or misaligned spindles. These results suggest that psoriasin expression does not have a biological effect on centrosome function as measured by a mitotic spindle morphology end-point.

ii. Microtubule Regrowth Assay

To additionally test if psoriasin has an effect on centrosome function *in vivo*, a MT regrowth assay was performed on three MDA-MB-231 clones (1a1, Fa3, and Fd3) whose psoriasin expression levels are describe above. Briefly, cells were grown on coverslips till they attained roughly ~60% confluency. Microtubules were then depolymerised in living cells with nocodazole (25uM) treatment and on ice for 2 hours. Coverslips were washed twice in cold PBS to remove nocodazole and subsequently placed into fresh warm media (37^0C) for exactly 5 minutes before fixation and IF processing (described above). Particular attention was paid to the regrowth time, as polymerisation of MT under these conditions is time dependent. In this way regrowth of microtubules from a naked centrosome takes place in living cells and is subject to the endogenous cytoplasmic environment.

The degree of MT regrowth was measured using a quantitative IF approach and is based on the assumption that the rate of MT elongation (polymerisation) is constant under experimental conditions (time and temperature kept constant). Therefore total fluorescence intensity centered on a centrosome is proportional to the amount newly generated MTs and is a function area size measured. Therefore MT regrowth was defined as the total fluorescent intensity from an area centered on the centrosome, minus the average background intensities for that same area, arising from both within the cell (where no tubulin polymerisation has occurred) and from outside the cell. In this way, background fluorescent intensities that are the result of intracellular and coverslip/slide variation are compensated for in the analysis.

Three independent MT regrowth experiments were performed, in which a total of 60 metaphase or anaphase cells presenting centrosomes with nascent nucleated MTs were quantified per cell line. If two centrosomes were in focus, the average fluorescent intensity was used for statistical analysis. Figure 6A shows the variation in the degree of MT regrowth observed; some cells have extensive regrowth (Figure 6A Top panels), in contrast to others with minimal regrowth (Figure 6A Top panels). However, there was no statistically significant difference ($p>0.05$; Anova with Dunnetts post-test) in the degree of MT regrowth from centrosomes when psoriasin is present (clones Fa3 and Fd3), or not (1a1) (Figure 6B). These results indicate that psoriasin does not influence centrosome function as defined by the capacity with which a centrosome can nucleate MTs.

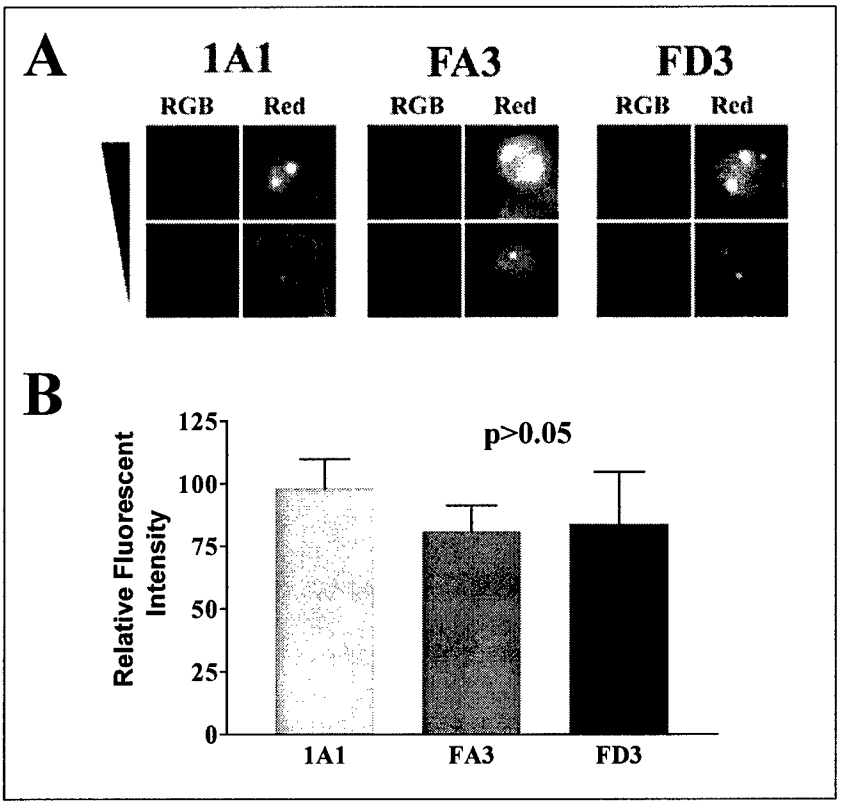

Figure 6: Psoriasin expression does not influence the capacity of centrosomes to nucleate microtubules as measured by a MT regrowth assay in MDA-MB-231 cells. (A) IF images to highlight the observed variability in the degree of MT regrowth and variability in the intracellular background contributed by unpolymerized tubulin dimers. Top panels show single cells with either one or two centrosomes with extensive MT regrowth, while bottom panels show cells with centrosomes having and minimal regrowth. (B) No statistically significant difference in the degree of MT regrowth is observed in MDA-MB-231 cells when psoriasin is expressed ($p>0.05$, Anova). Bars represent the Mean +/- S.D. (RGB, red green blue channels; Red, red channel)

Summary of key research accomplishments:

- His-tag does not interfere with His-hGCP3 ability to interact with γ-tubulin by co-immunoprecipitation assays.
- Robust His-hGCP3 expression has an immediate cytotoxic effect in transfected cells, but modest expression is tolerated at least 72 hours post-transfection.
- Psoriasin antibodies tested are not suitable for IF labelling therefore preventing co-locolisation studies with hGCP3.
- Psoriasin and His-hGCP3 cannot be consistently co-IP'ed suggesting that if an interaction exists that it may be weak or that a cell-cycle dependent interaction exists.
- Psoriasin expression does not influence centrosome function in epithelial breast cancer cells as determined by two assays for centrosome function: a microtubule regrowth assay and a mitotic spindle morphology assay.

In Progress:

- Generating an inducible His-hGCP3 model in MCF7 to study the effects of His-hGCP3 overexpression on centrosome function and influence on cellular properties associated with cancer progression.

Reportable outcomes:

1. Abstract: Biochemistry and Medical Genetics Departmental Seminar (Feb16, 2005)
2. Presentation: Biochemistry and Medical Genetics Departmental Seminar (Feb16, 2005)

Conclusion:

We conclude that the His-hGCP3 expression construct is suitable for this project. This is based the expressed tagged-protein's ability to retain γ-Tubulin interacting properties. Although immediate cytotoxic effects are observed, we would argue that co-IP assay are occurring in viable cells since a significant portion of adherent transfected cells retain persistent His-hGCP3 expression even at 72hr post transfection. Nevertheless, I have been unable to consistently co-IP psoriasin and His-hGCP3. Therefore, it is concluded that these two proteins either do not interact, or that an interaction cannot be detected under the experimental conditions used. This is consistent with results obtained from assays for centrosome function, showing that psoriasin overexpression in epithelial breast cancer cells does not influence overall mitotic spindle morphology or the capacity of centrosomes devoid of MTs to reinitiate MT nucleation in standard 2-D culture conditions. Future work that I am considering is to also test for an functional interaction at specific phases of the cell cycle, when other proteins that may possibly be important for the interaction may be expressed, and to test the effect of psoriasin on centrosome function under stress conditions.

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Appendices

Abbreviations used in the text:

Co-IP:	co-immununoprecipitation
Dapi:	4', 6-Diamino-2-phenylindole dihydrochloride
Dox:	doxycycline
GCP:	gamma-tubulin complex protein
γTuRC:	gamma-tubulin ring complex
hGCP3:	human gamma-tubulin complex protein 3
His-hGCP3:	histidine-tagged hGCP3
IF:	immunofluorescence
IgG:	immunoglobulin G
MM:	mouse monoclonal
MSM:	mitotic spindle morphology
MT:	microtubule
RP:	rabbit polyclonal
SOW:	statement of work
Tet-On:	tetracycline-on